

**EXHIBIT 3**

## Conservation and Variation in *Orthopoxvirus* Genome Structure

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### SUMMARY

*Orthopoxvirus* DNA from representative strains of rabbitpox, vaccinia, monkeypox, variola, cowpox and ectromelia viruses was analysed by cleavage with restriction endonucleases *Hind*III, *Xho*I or *Sma*I. Genome mol. wt. vary from about  $120 \times 10^6$  for rabbitpox to about  $145 \times 10^6$  for cowpox. Physical maps of cleavage sites are similar and characteristic for strains of the same *Orthopoxvirus* type. The distribution of *Hind*III sites suggests that an internal region of mol. wt. about  $30 \times 10^6$  is highly conserved between *Orthopoxvirus* genomes although some type-specific differences occur within this region, especially with strains of ectromelia virus. Conservation of internal sequences is less marked following analysis with *Xho*I although cleavages within this central region of particular genomes appear to represent a subset of preferred sites. Endonuclease *Sma*I cleaves exceptionally infrequently and distinguishes variola, monkeypox, vaccinia, cowpox or ectromelia viruses. Type specific differences result largely from extensive, near terminal variations in length and sequence.

Representative *Orthopoxvirus* genomes have rapidly renaturing terminal restriction fragments confirming the presence of near terminal, covalent cross-links. Terminal restriction fragments from the same or different genomes generally cross hybridize indicating the presence of near terminal repetitions of mol. wt. up to  $6 \times 10^6$  and which share at least a subset of common sequences. Variola strains however, appear to lack such sequences from one specific terminus which maps shorter than that of related viruses.

### INTRODUCTION

Restriction endonucleases have been valuable in analysis of the genome structure and organization of DNA viruses and have facilitated correlation between physical maps and the products of gene expression. Papovaviruses, adenoviruses and herpesviruses have been investigated extensively by physical mapping of normal and variant genomes (Brockman *et al.* 1973; Pettersson *et al.* 1973; Clements *et al.* 1976; Wilkie, 1976; Fried & Griffin, 1977; Werner & Zur Hausen, 1978) or of intertypic recombinants (Sambrook *et al.* 1975; Grodzicker *et al.* 1977; Marsden *et al.* 1978), by transcriptional mapping to restriction fragments (Sambrook *et al.* 1975; Pettersson *et al.* 1976; Clements *et al.* 1977) and by marker rescue with mapped DNA restriction fragments (Lai & Nathans, 1974; Miller & Fried, 1976; Arrand, 1978; Stow *et al.* 1978). Restriction endonuclease analysis has been extended to poxviruses despite the size and complexity of the genome (Gangemi & Sharp, 1976; Jaureguiberry, 1977; Wittek *et al.* 1977; Esposito *et al.* 1978; McCarron *et al.* 1978; Müller *et al.* 1978; Wittek *et al.* 1978a) in the obvious anticipation that analogous develop-

ments will follow. A previous paper from this laboratory describes the characterization of cowpox white pock variants as deletion mutants (Archard & Mackett, 1979) and the present report concerns some conserved features of *Orthopoxvirus* genome structure and indicates the nature and extent of variation between types.

#### METHODS

Methods were generally as described previously (Archard & Mackett, 1979) with the following variations:

*Virus growth and purification.* The following viruses were titrated and propagated by growth on the chorioallantois of 12-day, fertile hens' eggs: rabbitpox strain Utrecht; vaccinia strains Lister (Elstree), DIE, Hall Institute (HI) and Western Reserve (WR); monkeypox strains Denmark, España and Congo (human); cowpox strains Austria, Brighton, Ruthin and Daisy; ectromelia strains Hampstead and Moscow. Variola strains Butler (minor) and Harvey (major) were propagated in monolayer cultures of HeLa cells. Extraction and purification of viruses was essentially as described previously (Joklik, 1962; Archard & Mackett, 1979) with the further modification that viruses were sedimented through columns of 36% sucrose in 5 mM-citrate-phosphate buffer (McIlvain's), pH 7.4, overlaid with 10% dextran T10 solution as described by Esposito *et al.* (1978).

*Extraction of DNA.* Purified virus preparations were lysed initially at 4 °C for 30 min in 50 mM-tris-HCl, pH 7.8, 1 mM-EDTA, 27% sucrose, 1% sodium n-lauryl sarcosinate and 100 mM-2-mercaptoethanol before addition of proteinase K (500 µg/ml final concentration) and incubation at 37 °C until clear (J. Esposito, personal communication). Deproteinized DNA was recovered by precipitation at -20 °C with 2.5 vol. of ethanol and redissolved in 10 mM-tris-HCl, pH 7.5, 1 mM-EDTA.

*Cleavage of DNA.* DNA (1 µg in 25 µl final volume or *pro rata*) was cleaved with endonuclease *Sma*I [Boehringer Corp. (London), Lewes, Sussex] by incubation for 30 min at 30 °C in 30 mM-tris-HCl, pH 9, 3 mM-MgCl<sub>2</sub>, 15 mM-KCl. Conditions for cleavage with *Hind*III or *Xho*I were as described previously. Conditions for *Hind*III/*Sma*I or *Xho*I/*Sma*I double digests were as above.

*In vitro labelling of DNA restriction fragments by nick translation.* Hybridization probes were obtained often by nick translation (Rigby *et al.* 1977) of total DNA after endonuclease cleavage in nick translation buffer and subsequent separation of the <sup>32</sup>P-labelled restriction fragments in agarose slab gels. In such cases, DNA bands excised directly from gels were denatured by heating to 100 °C in 0.1 M-NaOH and after neutralization with an equivalent of HCl in the presence of 100 mM-tris-HCl, pH 7.5, were hybridized to separated restriction fragments immobilized on nitrocellulose membrane filters as described previously (Southern, 1975; Jeffreys & Flavell, 1977). In many experiments, non-labelled restriction fragments from various DNA preparations were separated simultaneously by electrophoresis in adjacent tracks of agarose slab gels and transferred to single nitrocellulose membrane filters for hybridization with individual probes.

#### RESULTS

##### *Cleavage of DNA*

Restriction fragments resulting from cleavage with endonuclease *Hind*III, *Xho*I or from *Hind*III/*Sma*I or *Xho*I/*Sma*I double digests are shown after separation by electrophoresis in 0.6% agarose slab gels (Fig. 1, 2 and 3, respectively). Fragments present in greater than 1 M amounts were identified by enhanced staining with ethidium bromide. Mol. wt. of

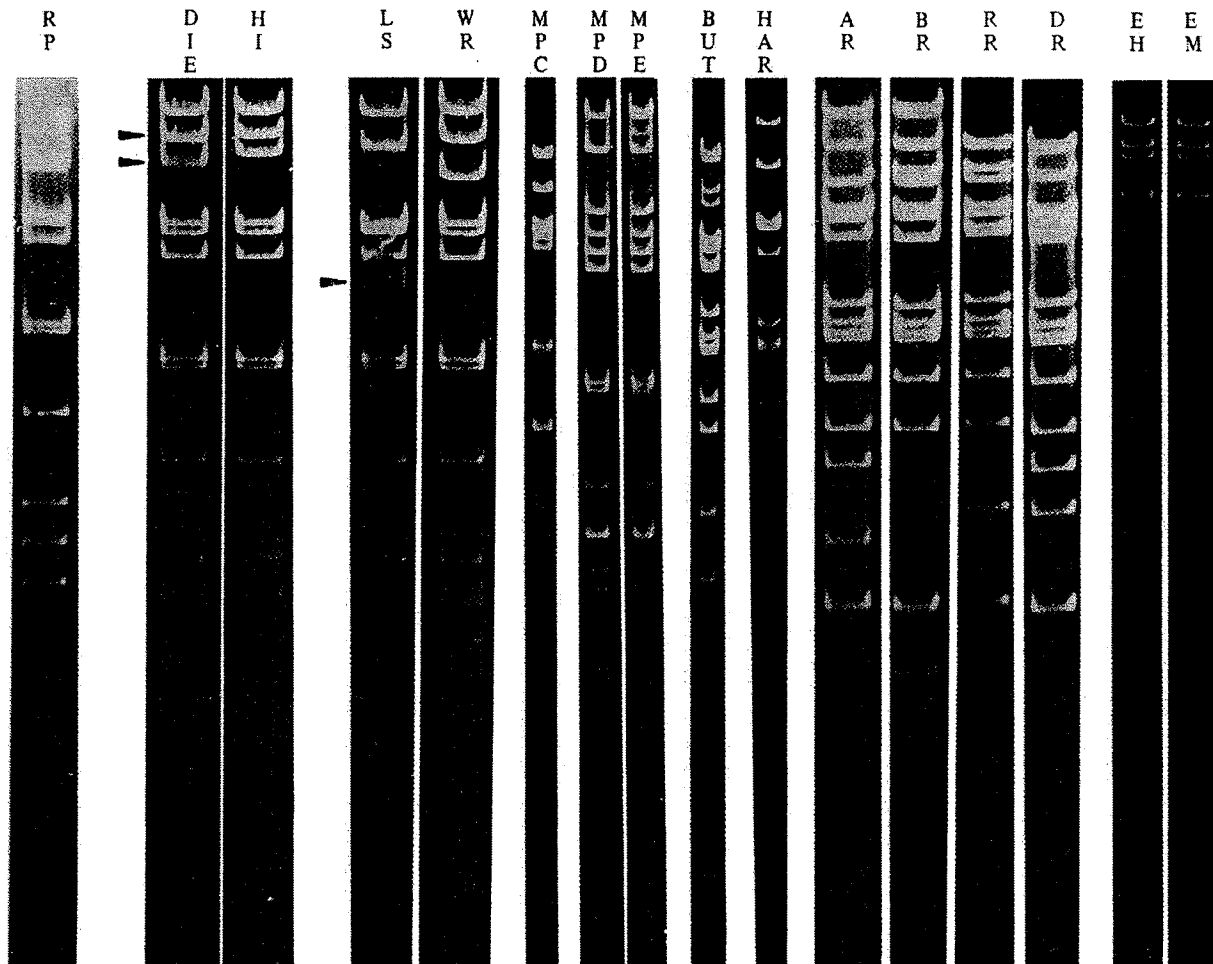


Fig. 1. *Hind*III restriction fragments of DNA from rabbitpox strain Utrecht (RP); vaccinia strain DIE, Hall Institute (HI), Lister (LS) or Western Reserve (WR); monkeypox strain Congo (MPC), Denmark (MPD) or España (MPE); variola strain Butler (BUT) or Harvey (HAR); cowpox red strain Austria (AR), Brighton (BR), Ruthin (RR) or Daisy (DR) and ectromelia strain Hampstead (EH) or Moscow (EM) separated by agarose slab gel electrophoresis. Note the size heterogeneity in DIE fragments B and C and Lister fragment G (arrowed).

fragments were estimated by comparison of electrophoretic mobilities with those of adenovirus type 2 or phage  $\lambda$  DNA restriction fragments of known mol. wt. as described previously (Tables 1 and 2). Fragments are lettered in order of decreasing size and total mol. wt. of the *Orthopoxvirus* genomes derived by summation. Subsets of restriction fragments from various DNA preparations co-migrate in electrophoresis and some fragments are present in digests of each *Orthopoxvirus* genome examined. For example, DIE/*Hind*III fragments G, H, I, L, M and O or DIE/*Xho*I fragments H and L have co-migrating partners in the corresponding digests of all DNA preparations suggesting the presence of sequences common to all genomes.

Wittek *et al.* (1978a) have demonstrated that terminal length heterogeneity in the DNA of vaccinia virus is eliminated on cloning the virus. We have observed similar heterogeneity in the length of certain *Orthopoxvirus* DNA restriction fragments (Fig. 1, vaccinia strain DIE/*Hind*III B and C, vaccinia strain Lister/*Hind*III G and Fig. 2, DIE/*Xho*I D and G, Lister/*Xho*I F; arrowed) and confirm that this is eliminated on cloning.

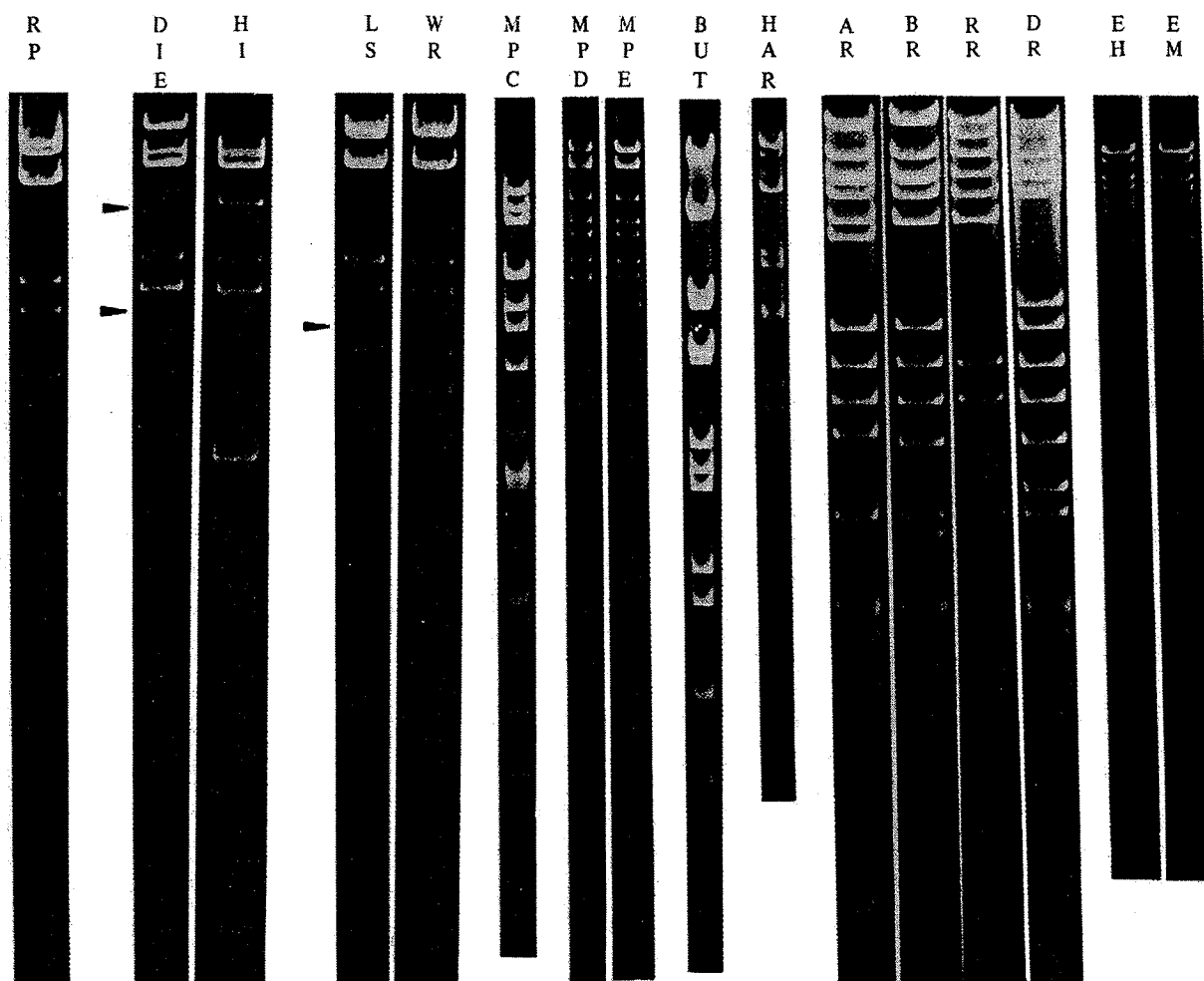


Fig. 2. *Xho*I restriction fragments of DNA from rabbitpox strain Utrecht (RP); vaccinia strain DIE, Hall Institute (HI), Lister (LS) or Western Reserve (WR); monkeypox strain Congo (MPC), Denmark (MPD) or España (MPE); variola strain Butler (BUT) or Harvey (HAR); cowpox red strain Austria (AR), Brighton (BR), Ruthin (RR) or Daisy (DR) and ectromelia strain Hampstead (EH) or Moscow (EM) separated by agarose slab gel electrophoresis. Note the size heterogeneity in DIE fragments D and G and Lister fragment F (arrowed).

#### *Mapping of restriction fragments*

We have mapped the *Hind*III sites in DNA from vaccinia strain DIE by hybridization of mapped restriction fragments of vaccinia strain Lister DNA (Wittek *et al.* 1977), <sup>32</sup>P-labelled by nick translation, to unlabelled, immobilized fragments from vaccinia DIE. The vaccinia Lister *Hind*III fragment D hybridizes specifically with the vaccinia DIE *Hind*III terminal fragment B. All other Lister *Hind*III fragments except Q and the terminal fragments G and B co-migrate and cross hybridize with DIE fragments (data not shown). *Hind*III sites in other vaccinia strains were mapped similarly. Vaccinia DIE or other mapped restriction fragments were used as hybridization probes to map the *Hind*III sites in related *Orthopoxvirus* genomes as described previously (Archard & Mackett, 1979) with the general assumption that cross hybridizing fragments represent equivalent regions in the genomes of closely related viruses. *Hind*III maps were confirmed and *Xho*I maps constructed by hybridization between fragments cleaved by either endonuclease from the same DNA and resulting in small linkage groups. Many *Xho*I maps for closely related viruses were confirmed

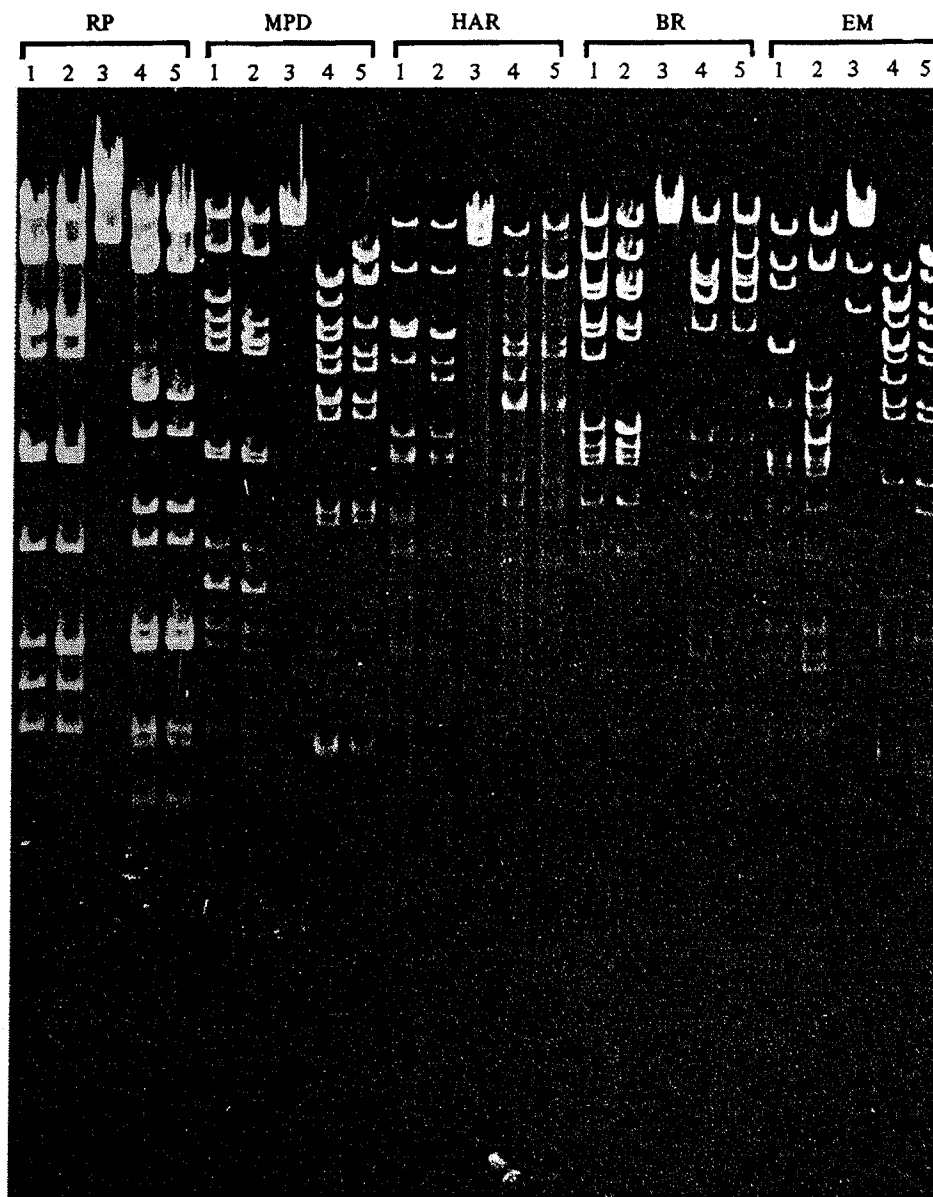


Fig. 3. (1) *Hind*III, (2) *Hind*III/*Sma*I double digest, (3) *Sma*I, (4) *Xho*I/*Sma*I double digest or (5) *Xho*I restriction fragments of DNA from rabbitpox strain Utrecht (RP), monkeypox strain Denmark (MPD), variola strain Harvey (HAR), cowpox red strain Brighton (BR) or ectromelia strain Moscow (EM) separated by agarose slab gel electrophoresis.

by cross hybridization of fragments from homologously restricted DNA preparations. Extensive tabulations of cross hybridizing fragments are not presented but a typical experiment testing hybridization between fragments of variola and vaccinia or monkeypox DNA is illustrated by the autoradiograph shown in Fig. 4. The specific hybridizations are summarized in Table 3. Generally, hybridizations to restriction fragments of mol. wt. less than about  $10^6$  were not tested but occasionally, tentative map locations have been assigned on the basis of co-migration (cowpox strain Brighton/*Hind*III Q) or by summation of mol. wt. (Brighton/*Hind*III R or S). In contrast to a previous report (Jaureguiberry, 1977), we found that endonuclease *Sma*I cleaves *Orthopoxvirus* DNA infrequently, often generating very

Mol. wt.  
x 10<sup>-6</sup>[illegible]



Table 2. *Mol. wt.\* of XhoI restriction fragments of DNA from strains of rabbitpox, vaccinia, monkeypox, variola, cowpox and ectromelia†*

Mol. wt. × 10 <sup>-4</sup>	RP	DIE	HI	LS	WR	MPC	MPD	MPE	BUT	HAR	AR	BR	RR	DR	EH	EM
40	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
37	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
31.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
27	A	A	—	A	A	—	—	—	A	—	A	A	—	A	—	—
26.5	—	—	—	—	—	—	—	—	—	—	—	—	B	—	—	—
25	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
24	B	—	—	B	B	—	—	—	—	—	—	—	—	—	—	—
22	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
18	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
17.4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
16.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
14.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
14	CC	CC	CC	CC	CC	—	—	—	BB	BB	C	C	D	CC	C	C
13.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
12	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11.8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10.8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9.8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9.6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9.4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9.2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8.7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8.1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
7.3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
7.2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6.9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6.8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6.4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6.3	E	FF	FF	E	E	—	G	—	—	—	—	G	—	F	I	—



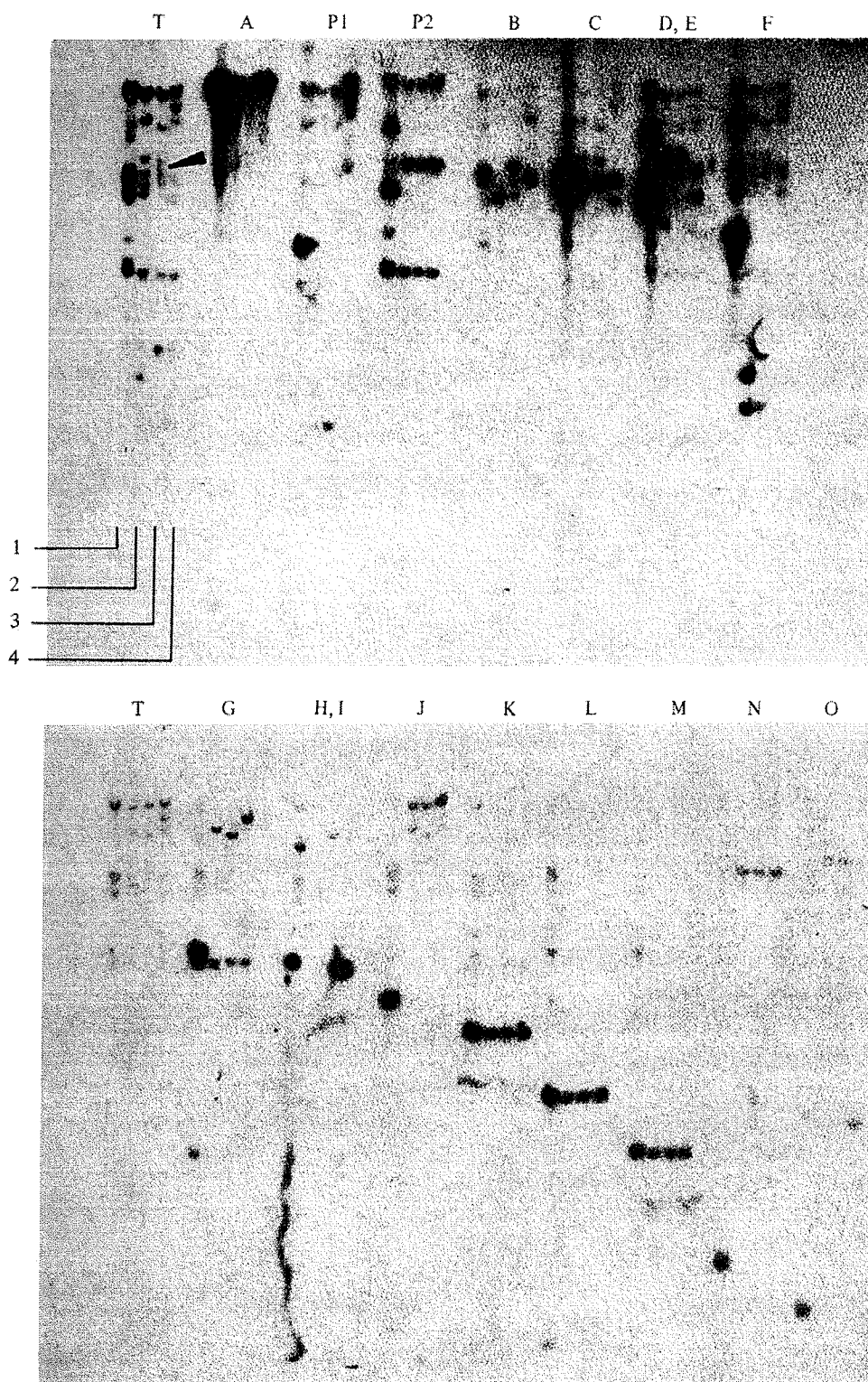


Fig. 4. Hybridization of  $^{32}\text{P}$ -labelled total DNA (T) or  $^{32}\text{P}$ -labelled *Hind*III restriction fragments A to O and two partial digest products (P1, P2) of DNA from variola strain Butler to immobilized *Hind*III restriction fragments of DNA from variola strain Butler (1), monkeypox strain Denmark (2), monkeypox strain Congo (3) or vaccinia strain Hall Institute (4). Each  $^{32}\text{P}$ -labelled probe is hybridized to an identical set of transfers. P1 = Butler/*Hind*III fragments C and F, P2 = Butler/*Hind*III fragments E and I and P3 (arrowed) = monkeypox Congo/*Hind*III F and M.

Table 3. Summary of hybridizations of <sup>32</sup>P-labelled *Hind*III restriction fragments A to O and partial digest products P1 and P2 of DNA from variola strain Butler to *Hind*III restriction fragments of DNA from variola, monkeypox or vaccinia viruses\*

Butler/ <i>Hind</i> III <sup>32</sup> P-probes	Immobilized <i>Hind</i> III restriction fragments			
	BUT	MPD	MPC	HI
A	A	A	A	A
P1†	P1, C, F, (A)‡	J, K, (A)	I, J, (A)	B, C, (ADE)
P2†	P2, D/E, H/I, (A)	D/E, G/H, (A)	D/E, G/H, (A)	D/E, G/H, (A)
B	B	F, N	P3‡, F, M, E§	C, D/E
C	C, P1, D/E	E, B, D	E, B, D	D/E, B
D/E	P2, D/E	C, D/E	C, D/E	F, D/E
F	P1, F	J, K	I, J	B, C, (ADE)
G	G, H/I	B, G/H	B, G/H	B, G/H
H/I	P2, H/I	G/H	G/H	G/H
J	J	A	A	A
K	K	I	I	I
L	L	L	K	J
M	M	M	L	L
N	N	D	E	D
O	O	C	C	K

\* Strains are as follows: variola strain Butler (BUT), monkeypox strain Denmark (MPD), monkeypox strain Congo (MPC) and vaccinia strain Hall Institute (HI).

† P1 = Butler *Hind*III F+C, P2 = Butler/*Hind*III E+I and P3 = MPC/*Hind*III F+M.

‡ Letters in parentheses indicate that hybridization was not significant.

§ Letters in bold type indicate that hybridization was due to contamination of probe with adjacent fragment.

large fragments. These were located initially by double digestion of mapped *Hind*III fragments and consideration of *Xho*I/*Sma*I double digests which permits unambiguous orientation of *Hind*III/*Sma*I cleavage products. For example, *Sma*I cleaves rabbitpox DNA at a single site, generating two fragments (Fig. 3). The rabbitpox *Hind*III/*Sma*I double digest shows that *Hind*III fragment A is shortened and a single, additional fragment is generated. The rabbitpox *Xho*I/*Sma*I double digest shows that *Xho*I fragment B is shortened to co-migrate with *Xho*I fragment C and a single, additional fragment is generated. The mol. wt. of the double digest products are compatible with one locus only for the single *Sma*I site. The allocation of terminal fragments was confirmed by rapid renaturation (see below). Physical maps of cleavage sites for *Hind*III and *Sma*I are shown in Fig. 5 and for *Xho*I and *Sma*I in Fig. 6. Hybridization data indicate that the apparent monkeypox strain Congo *Hind*III fragment D in Fig. 1 is a partial digest product representing Congo/*Hind*III fragments equivalent to monkeypox strain Denmark/*Hind*III fragments F and N; (Congo/*Hind*III F and M; Fig. 5). Table 1 takes account of this. *Hind*III maps for rabbitpox and vaccinia strain Lister (Elstree) have been presented previously (Wittek *et al.* 1977). The rabbitpox *Xho*I map agrees with further, unpublished data (R. Wittek, personal communication).

#### Confirmation of terminal restriction fragments by rapid renaturation

The termini of the vaccinia virus genome are cross-linked covalently (Geshelin & Berns, 1974) and renature rapidly on release from denaturing conditions (DeFilippes, 1976). This property has been exploited previously to identify the terminal restriction fragments of vaccinia or rabbitpox virus DNA (Wittek *et al.* 1977). We found that the terminal fragments of other *Orthopoxvirus* genomes behave similarly and in the present study were

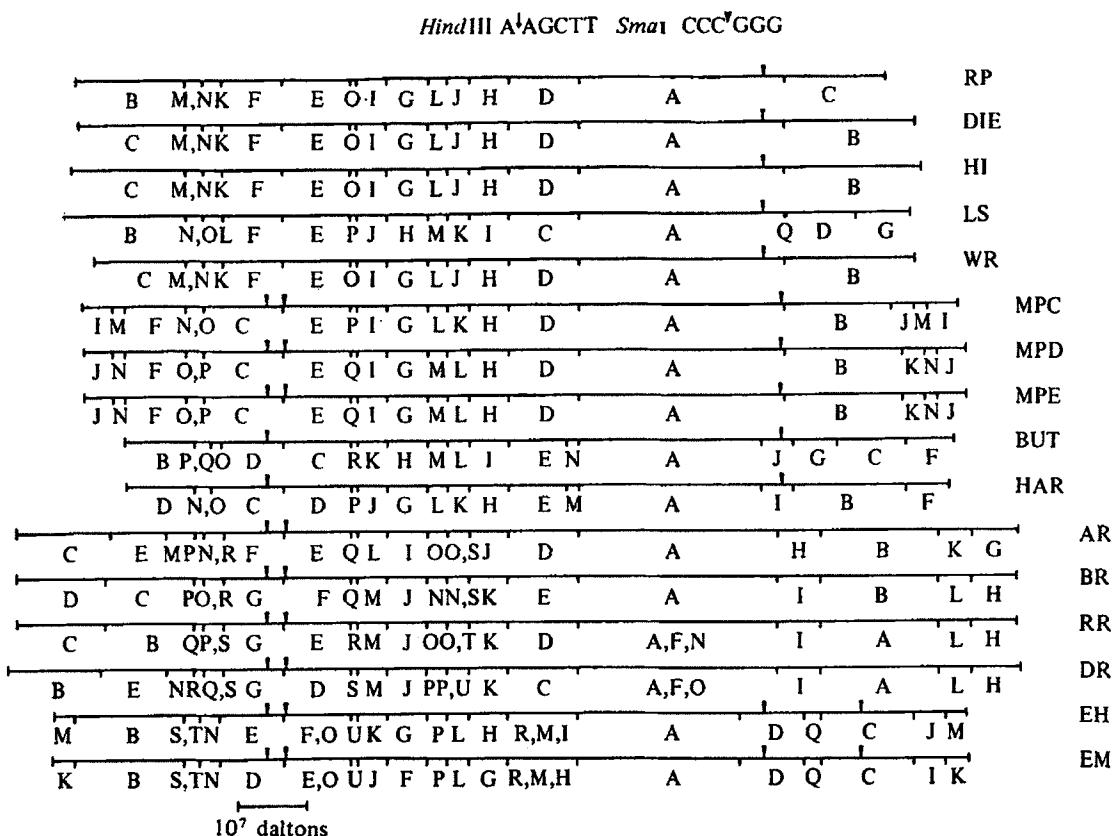


Fig. 5. Physical map locations of *Hind*III or *Sma*I restriction fragments of DNA from rabbitpox strain Utrecht (RP); vaccinia strain DIE, Hall Institute (HI), Lister (LS) or Western Reserve (WR); monkeypox strain Congo (MPC), Denmark (MPD) or España (MPE); variola strain Butler (BUT) or Harvey (HAR); cowpox red strain Austria (AR), Brighton (BR), Ruthin (RR) or Daisy (DR) and ectromelia strain Hampstead (EH) or Moscow (EM). Map locations of *Hind*III restriction fragments of DNA from rabbitpox or vaccinia strain Lister are from data of Wittek *et al.* (1977).

identified after formamide denaturation (Archard & Mackett, 1979) and co-electrophoresis with non-denatured digests (Fig. 7). Terminal restriction fragments (mapped by cross hybridization) of all the wild-type Orthopoxviruses tested were found to renature rapidly and thus appear to be naturally cross-linked. In some cases, for example *Hind*III digests of monkeypox or ectromelia DNA, co-migrating terminal fragments are cleaved from each end of the genome and only a single rapidly renaturing band is observed.

#### *Occurrence of terminal repetitive sequences in Orthopoxvirus genomes*

Wittek *et al.* (1977) described cross hybridization between the opposing terminal restriction fragments of rabbitpox virus DNA and showed subsequently, by partial restriction mapping of end-labelled terminal fragments, that the genomes of rabbitpox and vaccinia viruses contain inverted terminal repetitions and share a common subset of such sequences (Wittek *et al.* 1978a). In the present study, the occurrence of terminal repetitive sequences in various Orthopoxvirus genomes was revealed by cross hybridization between opposing terminal fragments and by hybridization to vaccinia strain Lister DNA terminal fragments. Either terminal fragment from vaccinia Lister hybridizes specifically to terminal fragments

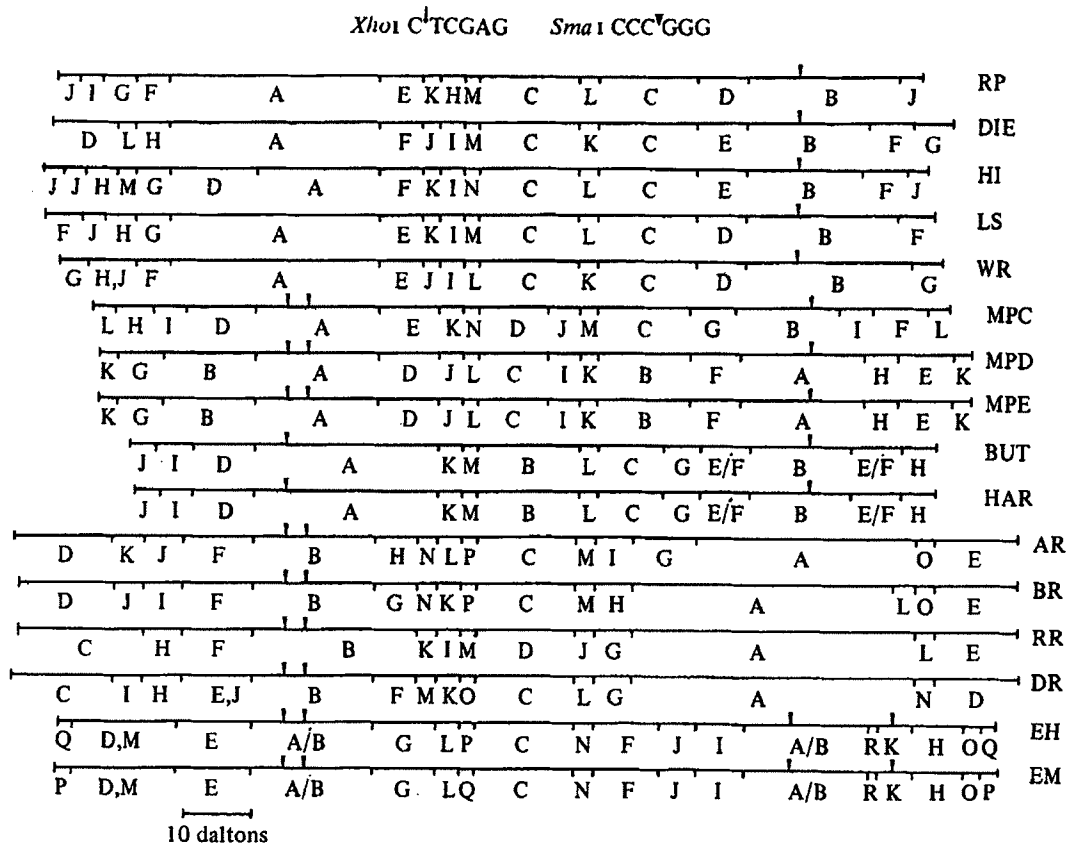


Fig. 6. Physical map locations of *Xho*I or *Sma*I restriction fragments of DNA from rabbitpox strain Utrecht (RP); vaccinia strain DIE, Hall Institute (HI), Lister (LS) or Western Reserve (WR); monkeypox strain Congo (MPC), Denmark (MPD) or España (MPE); variola strain Butler (BUT) or Harvey (HAR); cowpox red strain Austria (AR), Brighton (BR), Ruthin (RR) or Daisy (DR) and ectromelia strain Hampstead (EH) or Moscow (EM).

of rabbitpox, monkeypox, variola, cowpox and ectromelia viruses (Fig. 8). Endonuclease *Hind*III generates co-migrating fragments from the opposing termini of monkeypox or ectromelia viruses (Fig. 5) and thus appears to cleave within terminal repetitions. This is reflected by the extent of hybridization to these bands. Vaccinia strain Lister terminal fragment *Hind*III/G hybridizes weakly to variola strain Harvey *Hind*III terminal fragment F but not to the opposing terminal fragment Harvey/*Hind*III D. Additionally, variola strain Butler *Hind*III terminal fragment B has little sequence homology with its opposing terminal fragment *Hind*III F and, unlike Butler/*Hind*III F, fails to hybridize with the monkeypox strain Denmark *Hind*III terminal fragment J or the equivalent monkeypox strain Congo *Hind*III fragment I (Table 3). These data suggest that variola strains lack part if not all of a repetitive sequence at that terminus.



Fig. 7. Rapidly renaturing (1) or non-denatured (2) *Hind*III restriction fragments of DNA from vaccinia strain Hall Institute (HI), monkeypox strain Denmark (MPD), variola strain Butler (BUT), cowpox red strain Brighton (BR) or ectromelia strain Moscow (EM).

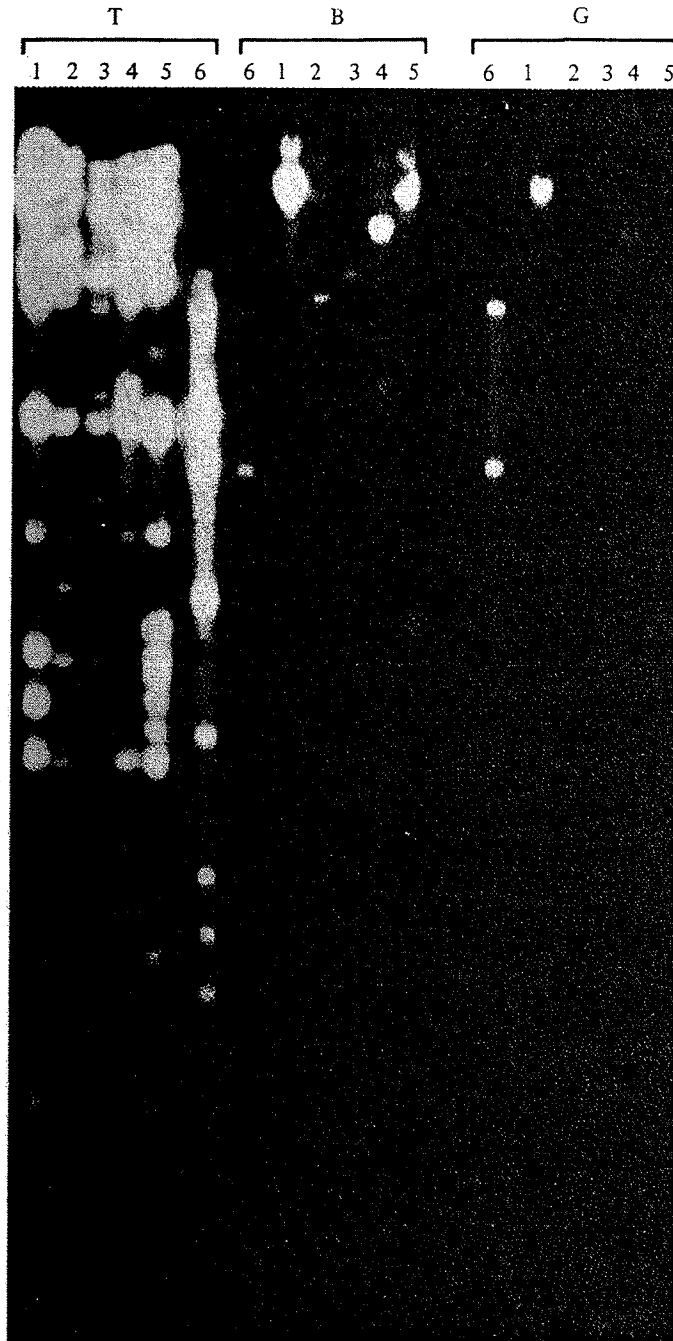


Fig. 8. Hybridization of  $^{32}\text{P}$ -labelled total DNA (T) or  $^{32}\text{P}$ -labelled *Hind*III restriction fragment B or G of DNA from vaccinia strain Lister to immobilized *Hind*III restriction fragments of DNA from rabbitpox strain Utrecht (1), monkeypox strain Denmark (2), variola strain Harvey (3), cowpox red strain Brighton (4), ectromelia strain Moscow (5) or vaccinia strain Lister (6).

## DISCUSSION

Restriction endonuclease analysis shows that the genomes of orthopoxviruses vary considerably in size. Variation in the apparent mol. wt. of particular genomes, derived by summation of the mol. wt. of restriction fragments produced by either *Hind*III or *Xho*I, is generally less than 5% and results mainly from the difficulty of estimating the size of large fragments. Rabbitpox, vaccinia and variola viruses have similarly sized genomes with strain average mol. wt. of  $120$ ,  $124$  and  $121 \times 10^6$ , respectively. The genomes of monkeypox, cowpox or ectromelia viruses are significantly larger with strain average mol. wt. of  $128$ ,  $145$  and  $136 \times 10^6$ , respectively. These values agree well with data of Müller *et al.* (1978) with respect to rabbitpox and vaccinia but not cowpox or ectromelia. The cowpox data presented here are consistent in cleavage of DNA from four strains with either endonuclease.

Physical maps for the cleavage sites of endonucleases *Hind*III or *Xho*I were constructed largely by hybridization between restriction fragments from different genomes with the assumption that cross hybridizing fragments represent equivalent regions of the DNA of closely related viruses. This assumption appears justified on the basis of hybridizations between mapped *Hind*III and *Xho*I fragments from the same genome, which would be predicted to overlap, hybridizations between co-migrating fragments cleaved from different genomes by the same endonuclease and by consistency in the locations of the rare *Sma*I sites determined by double digestion. The physical maps are similar for strains of the same *Orthopoxvirus* type but characteristic of types, often permitting identification by examination of the relative mobilities of restriction fragments. Differentiation on the basis of restriction patterns has been described previously (Esposito *et al.* 1978). Endonuclease *Hind*III cleaves large numbers of co-migrating and cross hybridizing fragments from the genomes of orthopoxviruses. A similar phenomenon was observed by Müller *et al.* (1978) and shown by physical mapping in the case of DNA from rabbitpox or vaccinia strain Lister (Elstree) to result from cleavages in an internal, conserved region of the genomes (Wittekk *et al.* 1977). The *Hind*III maps presented here indicate a high degree of sequence conservation in a central region of mol. wt. about  $30 \times 10^6$  in the genomes of a wide range of orthopoxviruses. Alignment of the physical maps, however, indicates several type specific differences even within the conserved region. Variola strains have an additional site which generates the Butler *Hind*III fragment N (Harvey/*Hind*III M) and cowpox strains a site which appears by summation to generate the  $0.62 \times 10^6$  mol. wt. fragment Brighton/*Hind*III S and the co-migrating fragment from other cowpox strains. Ectromelia strains have three additional, unique *Hind*III sites which generate fragments mapping at the edges of this region and thus show the least degree of internal sequence conservation. Conservation of internal sequences between *Orthopoxvirus* types is less obvious from physical maps of *Xho*I sites though several co-migrating and cross hybridizing fragments are cleaved by *Xho*I from all the genomes analysed. Cleavage within this central region of particular genomes appear to represent subsets of preferred sites, and type specific differences include the additional site which generates the monkeypox strain Congo *Xho*I fragment J and its co-migrating partners from monkeypox strains Denmark or España.

Type-specific differences in genome structure consist mainly of extensive near terminal variations in sequence and in length which presumably encode the distinguishable phenotypes. The increased size of cowpox virus genomes compared with those of vaccinia strains, for example, results from the presence of additional and in some cases unique, sequences at either terminus. In contrast, the variola strains map noticeably shorter than related viruses at one specific terminus. Endonuclease *Sma*I recognizes the sequence C C C G G G and cleaves *Orthopoxvirus* DNA (36% G C) exceptionally infrequently, often generating

very large fragments which are difficult to characterize. The distribution of *Sma*I sites, located by double digestion with *Hind*III or *Xho*I, is characteristic of *Orthopoxvirus* types and sites map co-linearly between strains when maps are aligned by common *Hind*III sites. Rabbitpox and vaccinia viruses have a single site which coincides with a site present also in ectromelia strains. Two other, adjacent sites in ectromelia viruses map co-linearly with sites present in cowpox and monkeypox viruses whilst a fourth is unique to ectromelia. Only one of this pair of *Sma*I sites common to all ectromelia, cowpox and monkeypox viruses examined is present in variola strains but a second site in variola coincides with a third site in monkeypox strains. *Sma*I digests therefore readily distinguish between vaccinia, monkeypox and variola viruses. In this context, the difference between monkeypox and variola strains is the loss of a single site.

The similarity between the genomes of strains of the same *Orthopoxvirus* type is marked but strain specific differences of two sorts are observed. Firstly, the loss or acquisition of small numbers of restriction sites as would be expected from single base changes deleting or, less commonly, generating a specific endonuclease recognition sequence. Consequently, sites at particular locations are often present in more than one strain, for example the site generating the *Hind*III fragment M from cowpox strain Austria or N from strain Daisy, although some apparently unique sites exist, for example that generating the cowpox strain Brighton *Xho*I fragment L. Secondly, variation in the length of fragments cleaved from apparently equivalent regions of the genomes as would be generated by relatively small DNA deletions or insertions, for example monkeypox strain Congo fragments *Hind*III or *Xho*I B, variola strain Harvey *Xho*I fragment I and ectromelia strain Hampstead *Hind*III fragment E. Such variation is especially common in the length of terminal restriction fragments, for example monkeypox strains Congo *Hind*III I (*Xho*I L) and Denmark *Hind*III J (*Xho*I K) or ectromelia strains Hampstead *Hind*III M (*Xho*I Q) and Moscow *Hind*III K (*Xho*I P). This should be distinguished from the length heterogeneity which accumulates in terminal restriction fragments as a result of passage of individual strains (Fig. 2; vaccinia strain DIE) and is eliminated on cloning (Wittek *et al.* 1978*b*) although the phenomena may be related in that successive, cloned isolates may have discrete terminal fragments of various lengths. A white pock variant of Brighton red cowpox has been shown previously to be a deletion mutant lacking about 12% of the wild-type genome including the cross-linked sequence from one specific terminus (Archard & Mackett, 1979).

The very close similarity between rabbitpox and vaccinia strains other than Lister is revealed by the presence of a single, characteristic *Sma*I site and by *Hind*III maps which show that variation is again confined to the length of the terminal fragments. Lister is unique amongst a total of seven vaccinia strains examined in possessing additional, near terminal *Hind*III sites.

The natural occurrence of near terminal, covalent cross-links in the DNA of vaccinia virus is well documented (Geshelin & Berns, 1974; De Filippes, 1976; Wittek *et al.* 1977). The present study indicates that such cross-links occur at each terminus of the genomes of representative wild-type orthopoxviruses, allowing the fact that some endonucleases generate co-migrating terminal fragments from the genomes of some types. Variola retains the cross-link at the terminus which maps shorter than related viruses.

Inverted terminal repetitions in the DNA of vaccinia and rabbitpox viruses have been characterized previously and shown to contain a subset of common sequences (Wittek *et al.* 1978*a*). We have reported the occurrence of near terminal repetitions in cowpox virus DNA which also have sequence homology with those of vaccinia although the presence of both sets of such sequences is not required for DNA replication (Archard & Mackett, 1979). The present report demonstrates that, with the exception of variola strains, unique, opposing

terminal restriction fragments from the genomes of particular orthopoxviruses cross hybridize strongly and hybridize also with terminal fragments from the genomes of other types. The opposing terminal fragments from variola viruses cross hybridize weakly and fragments mapping at one specific terminus fail to hybridize with terminal fragments of other *Orthopoxvirus* genomes. These data suggest that the terminal repetition of variola virus DNA is deleted at that terminus which maps shorter than DNA from related viruses and indicate that the genomes of all other representative wild-type orthopoxviruses examined contain such repetitions which share certain common sequences. The generation of particular, co-migrating restriction fragments from each terminus of certain genomes results presumably from cleavages within repetitions and suggests that the repetitive sequences overall are type-specific. The mol. wt. of the terminal repetitions in the genomes of the rabbitpox and vaccinia (Lister) have been determined as  $3.4$  to  $3.6 \times 10^6$  and  $7.4$  to  $8.0 \times 10^6$ , respectively (Wittek *et al.* 1978a). Data presented previously (Archard & Mackett, 1979) indicates that the size of the cowpox strain Brighton repetition is between  $6.3$  (2 M terminal fragment *Pst*I K) and  $6.6$  (terminal fragment *Hind*III H)  $\times 10^6$ . Minimum estimates of the sizes of some terminal repetitions can be made by consideration of the sizes of terminal fragments generated apparently by symmetrical cleavages within the repetitions. Thus, the monkeypox *Hind*III plus molar fragments I and M (Congo) or J and N (Denmark, Espana) give minimum estimates of  $6.1$  and  $5.6 \times 10^6$ , respectively, and the ectromelia *Hind*III fragments M (Hampstead) or K (Moscow)  $3.1$  and  $3.4 \times 10^6$ , respectively.

The data presented indicate that a central conserved region, terminal, covalent cross-links and terminal repetitions containing subsets of common sequences are general features of *Orthopoxvirus* genome structure. Conserved sequences code presumably for functions common to orthopoxviruses while individual characteristics are generated by near terminal sequences which show extensive type-specific variation. Physical maps are similar for strains of the same *Orthopoxvirus* type but characteristic of types and permit rapid identification including the important distinction between vaccinia, monkeypox and variola viruses. These data constitute a basis for finer mapping which no doubt will reveal further type-specific differences and facilitate genomic analysis of mutants and intertypic recombinants. In conjunction with phenotypic analysis this will permit the allocation of certain *Orthopoxvirus* gene functions to DNA loci.

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